Isotopic Labeling of Phosphatidylcholine in the Choline Moiety

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ABSTRACT

Two simple methods for the synthesis of phosphatidylcholine (lecithin) isotopically labeled in the methyl group of the choline moiety are described. Phosphatidylcholine is converted to phosphatidylethanolamine by enzymic transphosphatidylation, and this is methylated using either methyl iodide or diazomethane to give a product isotopically enriched in all three choline methyl groups.

The availability of radioactively labeled phospholipids is essential for many biochemical studies of biological membranes and lipid metabolism (1,2), and ^{13}C- and ^{2}H-labeled phospholipids are becoming increasingly important for biophysical studies of membranes (3,4). The phospholipid most used is phosphatidylcholine with a wide range of defined alkyl chains, and we describe two methods for labeling this lipid in the choline moiety. Stoffel et al. (5) have proposed a procedure that involves conversion of phosphatidylcholine to phosphatidyl-*N,N*-dimethylethanolamine using sodium benzene thiolate followed by methylation with labeled methyl iodide. Only one methyl group out of three is accessible to labeling by this method. Methylation of phosphatidylethanolamine was used by Stockton et al. (6); however, in order to achieve high yield, they needed to react with a large excess of labeled methyl iodide for 14 days under alkaline conditions which is likely to cause some hydrolysis of the alkyl chains. The procedures we report here also involve a methylation of phosphatidylethanolamine, but are rapid, mild, and efficient. We first describe the enzymatic preparation of phosphatidylethanolamine which we have found is most conveniently made from phosphatidylcholine utilizing the transphosphatidylation activity of phospholipase D (7).

Carbon or hydrogen isotopes are introduced from labeled methyl iodide by methylation using stoichiometric quantities of starting materials under mild conditions. Hydrogen isotopes are readily and more cheaply available as ^{2}H or ^{3}H water, and so we describe a second method allowing introduction of hydrogen isotopes with diazomethane in the presence of isotopically enriched water. These methods offer the advantage of maximum isotopic enrichment since all three methyl groups of choline head-group will be labeled. This is a particularly important point when preparing lipids for use in ^{13}C- and ^{2}H-nuclear magnetic resonance (NMR) where sensitivity is often the limiting factor.

EXPERIMENTAL PROCEDURES

Phosphatidylcholine

Pure synthetic 1,2-dimyristoyl *sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl *sn*-glycero-3-phosphocholine, and 1,2-dioleoyl *sn*-glycero-3-phosphocholine were prepared by the method of Robles and Van den Berg (8).

Phospholipase D

Partially purified phospholipase D (phosphatidylcholine phosphatidohydrolase EC 3.1.4.4) from cabbage was prepared by a modification of Yang's purification (9). Chopped inner cabbage leaves are homogenized in a blender with ca. 30 ml distilled water per 100 g cabbage, and the homogenate filtered by pressing through four layers of muslin and adjusted to pH 6.5 if necessary. Aliquots of 300 ml are heated rapidly in a 1-liter conical flask to 55 C in a boiling water bath, maintained for 5 min in a 55 C water bath, and then cooled rapidly in an ice bath. The precipitate is centrifuged (10 min, 10,000 rpm, MSE 18 6 x 300 rotor), and to the supernatant are added two volumes of acetone precooled to -15 C. The precipitate is centrifuged (30 min, 2000 rpm, MSE Major 4 x 1500 rotor) and resuspended in 2 mM potassium phosphate pH 6.8 and dialyzed for 3 hr into 1 liter 2 mM potassium phosphate pH 6.8. The dialysate is clarified by centrifugation (15 min, 20,000 rpm, Sorvall RC-5 8 x 50 rotor) and freeze-dried. In a typical preparation, about 6 kg Dutch White cabbage yielded 5.2 g enzyme.

Phospholipase D of similar activity is available commercially from Boehringer, Mannheim, Germany, and Sigma, St. Louis, MO.

Transphosphatidylation

Phospholipase D (50 mg) is dissolved in 100 ml 10% (v/v) ethanolamine, 40 mM calcium chloride adjusted to pH 5.6 with acetic acid. Dioleoyl glycerophosphocholine (500 Mg) dissolved in 100 ml diethyl ether, previously washed three times with equal volumes of water, is added to the aqueous phase and left stirring overnight. Dimyristoyl glycerophospho-

choline (500 mg) or dipalmitoyl glycerophosphocholine are dissolved in water-washed chloroform and stirred with the aqueous solution as above. (Water washing of solvents is essential to saturate the solvent with water and to remove any ethanol present which competes strongly with ethanolamine in the transphosphatidylation reaction.) The reaction is followed using thin layer chromatography (TLC) to detect the disappearance of phosphatidylcholine and appearance of phosphatidylethanolamine in the organic phase. On completion, the reaction is stopped by acidification to pH 1-2, and the lipid extracted with chloroform and dried. The crude product can be used directly for the methylation reactions since the major contaminant, phosphatidic acid, does not interfere with them. However, any residual phosphatidylcholine will reduce the degree of labeling in the final product, and the phosphatidylethanolamine can be purified by chromatography on silica gel is desired. The yield of phosphatidylethanolamine is typically 50-60% after purification.

Methylation with Methyl Iodide

Phosphatidylethanolamine (300 mg) is dissolved in 10 ml tetrahydrofuran and 2 g dry silver carbonate on Celite (Fetizon's reagent) (10) added in 10 ml acetonitrile. Labeled methyl iodide (0.2 g) is added, and the flask is stoppered and stirred for 3 hr in the dark at 35-40 C. The suspension is filtered through Celite, and the filter washed with a diethyl ether-methanol mixture. The filtrate is concentrated by rotary evaporation and purified by silica gel chromatography. Dipalmitoyl glycerophosphoethanolamine (300 mg) gave [N-methyl-^{13}C]-dipalmitoyl glycerophosphocholine (240 mg, 75% yield) which was identical to authentic material by TLC. As shown by mass spectrometry (MS) and ^{13}C-NMR, the product was isotopically enriched solely in the choline moiety and to the expected degree.

Methylation with Diazomethane

Phosphatidylethanolamine (50 mg) is dissolved in 1 ml dioxane, and 50 μl ^{3}H water is added. Diazomethane in diethyl ether (11) is added dropwise to maintain a yellow color in the solution over 1 hr. Careful exclusion of light is essential for methylation of unsaturated lipids by this method. The reaction is terminated by evapoaration of excess reagent, and the labeled phosphatidyl-choline purified by silica gel chromatography. Dipalmitoyl glycerophosphoethanolamine (50 mg) gave pure dipalmitoyl glycerophospho[N-methyl-^{3}H] choline (17.9 mg, 34%) pure, identical to authentic material by TLC and MS. Using tritiated water at about 3 Ci/ml (The Radiochemical Centre, Amersham), the specific activity was 5.0 x 10^{10} dpm/mmole, and 95% of the radioactivity ran with marker phosphatidylcholine in TLC. At least 98% of the radioactivity could be removed from the lipid fraction by digestion with phospholipiase D. Similar results were obtained for dimyristoyl glycerophosphocholine and dioleoyl glycerophosphocholine. The efficiency of hydrogen isotope exchange of diazomethane under these conditions is low; however, this is amenable to improvement (13).

Purification and Analysis

Lipids were purified by chromatography on silica gel. Oven-dried silica AR (Mallinckrodt CC-4) at 100 mg/mg lipid is poured in a column in chloroform, and crude lipid loaded in chloroform. Lipids are eluted with chloroform containing increasing proportion of methanol, and the fractions are followed by TLC. Phosphatidic acid elutes at 2-5% methanol, phosphatidylethanolamine at 15-20%, and phosphatidylcholine at 70-80%.

TLC on silica gel plates was carried out using three solvent systems: (a) chloroform-methanol-water-ammonia solution, 65:30:3:1; (b) chloroform-methanol-acetic-acid-water, 75:25:8:3; (c) chloroform-methanol-water, 65:30:4. Fractions were visualized with iodine vapor or Dittmer's spray for phosphorus (12).

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